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Histopathological Effects of the *Annona muricata* Aqueous Leaves Extract on the Liver and Kidneys of Albino Mice

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Abstract

Annona muricata (AM) is a plant that is traditionally used for its various medicinal benefits. The present investigation aims at estimating the anti-inflammatory activity of AM leaf extract against peritonitis induced by *Bacillus furmis*. Albino mice (25-30 grams) were intra-peritoneally injected with 1.5×10^9 CFU Bacillus furmis and treated with either AM water extract (100 mg/kg) or combination of AM and aspirin. Control groups received only water. Mice were pre-treated with AM extract either two hours or 7 days prior to bacterial infection. Animals were sacrificed three days after bacterial infection. Livers and kidneys were removed and preserved in 10% formalin solution for histopathological studies or in Carnoys solution to assess level of glycogen in liver. The histopathological studies of animals treated with 100 mg/kg AME and 100 mg/kg aspirin showed a synergistic anti-inflammatory effect. However, the leaf extract alone exhibited different effect depending on the dose used. At high doses, AM leaf extract caused a toxic effect on both the liver and the kidney of mice, whereas at low dose it exhibited a protective effect. Therefore, we conclude that *Annona muricata* extract should be taken with precaution if to be used for extended period of time.

Keywords: Inflammation; *Annona muricata*; Liver glycogen; Kidney

Introduction

Inflammation is the body's immediate reaction to injury inflicted on its tissues and cells by pathogens, chemicals, or physical damages [1]. It is characterized by five cardinal signs, namely redness, swelling, hotness, pain and loss of function. Inflammation can be classified into acute inflammation and chronic inflammation [2]. Acute inflammation is a short-term response that may be healed through leukocytes infiltration to the damaged region. Removing the stimulus leads to repair of the damaged tissue. However, chronic inflammation is active, prolonged, uncontrolled and maladaptive response that leads to tissue destruction. Failure to improve the inflammatory response may lead to chronic inflammation and sepsis [3,4].

Sepsis constitutes a major cause of morbidity and mortality. It leads to multiple organ dysfunction syndromes, multiple organ damage and septic shock [5,6].

Liver plays an essential role in removing Gram-positive bacteria such as bacillus and their exotoxin from the bloodstream [7] and hence eliminate risk of bacteremia. The potential importance of hepatic exotoxin detoxification is suggested by evidence their ability to inhibit hepatic mitochondrial fatty acid oxidation in rat livers and causing liver failure [8]. Besides the liver, which is considered the key organ in drug metabolism, the kidneys contribute to drug elimination through their excretion. Detoxification of drug metabolites can induce various and varying degrees of injury to the liver, and accumulation of toxin in kidney lead to kidney failure [9,10].

Annona muricata is a member of the Annonaceae family and is an evergreen tree. It is cultivated in tropical and subtropical regions and considered as a traditional medicine [11]. Its leaves contain several groups of substances collectively called annonaceous acetogenins that include murihexocin, annocuricin [12] annopentocin A, B and C, (2,4-cis)-annomuricin-D-one, murihexocin A and B, (2,4-trans)-annomuricin-D-one, 4-acetyl gigantetrocin, cis-gigantrionin [13] muricatocin A, B and C [14] and annohexocin [15]. These compounds are highly potent and selective against microbial resistance [16]. They showed anti-tumor effects *in vivo* and *in vitro* [17-19]. The essential oils of *A. muricata* leaves have parasiticidal, anti-diarrheal, rheumatological, and anti-neuralgic properties [20,21]. The leaves extracts are gastroprotective [22], anti-diabetic, hepatoprotective [23] anti-bacterial [24], anti-arthritic, anti-inflammatory [25] and are modulators of the innate immune system [26].

AM water extract is commonly used in folk medicine to reduce inflammation as synthetic drugs have many side effects on liver and kidneys. The aim of this paper is to investigate the effect of AM extract against inflammation caused by a newly identified bacterial strain in our laboratory in albino mice.

Materials and Methods

Preparation of the water extract of *Annona muricata* leaves

Sterilized and shade air-dried *Annona muricata* leaves were purchased from USA from Nallife Company. AM leaves were of Thailand origin. Sixty grams of powdered AM leaves were soaked in 200 ml of boiling distilled water. Filtrate were extracted a second time with an additional 200 ml of boiling distilled water. The combined filtrate was then freezed-dried yielding a 2.43 g of brown, sticky precipitate that was stored at 4°C. Aliquot of extract residue were weighed and suspended in de-ionized distilled water to a final concentration of 34.2 mg/ml before use.

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Animals

Healthy male albino mice BABL/c weighing 25-30 g obtained from the animal house of BAU were used in this study. The animals were housed under standard laboratory conditions of light, temperature and humidity with a 12 h light/dark cycle and had access to food and water *ad libitum* throughout the study. Mice were randomly divided into seven experimental groups of 3 mice each. Each group received the treatment according to Table 1. The animals were sacrificed 3 days after treatment, and then both liver and kidney were removed for histological studies.

Ethical consideration

In this study, the experimental procedures performed on mice were approved by the IRB Ethics Committee of Beirut Arab University. The animals were taken care of in accordance with the recommendations provided in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences.

Induction of peritonitis

Peritonitis was induced by injecting 1.5×10^9 CFU of *B. firmus*. Bacteria were cultured in Luria Bertani (LB) broth at 37°C. They were harvested at the mid-log phase and washed twice with sterile saline before injection.

Histological techniques

Paraffin tissue processing and staining:

Hematoxylin and eosin staining: Pieces of the isolated kidneys and the livers were washed, fixed using 10% formaldehyde for 18 h, dehydrated through series of graded alcohol, cleared in xylene and embedded in molten paraffin wax. Tissue blocks were sectioned 4 µm thickness using microtome (14274 microtome HM340E), deparaffinized and stained with Heamatoxylin and Eosin. The sections were examined with a Zeiss Primo light microscope with axioVision software (400x magnification) and photomicrographed using AxioCam camera for analysis.

Best carmine staining: Pieces of isolated liver were washed, fixed in Carnoy's solution for one and half hours, wash in 100% ethanol, cleared in xylene, and embedded in molten paraffin wax. Tissue blocks were sectioned at 6 µm thickness de-paraffinized and stained with the best carmine for glycogen detection.

Congo red staining: Pieces of the isolated kidney and liver tissues were treated similar to hematoxylin and eosin but stained with hematoxylin and A Congo red to check for amyloidosis. The sections were examined with the light microscope at 400x magnification and photomicrographs of the sections were taken for further analysis.

Results

Histopathology of the livers of animals in group1 showed typical hepatolobular architecture, consisting of a central vein with radiating cords of hepatocytes separated by sinusoids. The hepatocytes are polygonal in shape, with central, lightly stained nuclei and prominent nucleolus. Few binucleated cells are also present. The cytoplasm is regularly distributed (Figure 1).

On the other hand, the three groups of mice treated with 100 mg/kg AME (group 2) for 7 days, or infected with 1.5×10^9 cfu of *B. firmus* (group 3), or infected with 1.5×10^9 cfu of *B. firmus* 7 days post AME treatment (group 5) showed deranged architecture of hepatocyte with global moderate hydropic degeneration. Nucleoli were not clearly seen with multiple binucleated cells and deranged sinusoidal arrangement with dilatation and vascular congestion (VC). Accumulation of lymphocytes (L) around the central vein indicates inflammation. Hepatic necrosis (NC) is observed through pyknotic or disappeared nuclei (Figures 2-4). Hyalinosis accumulating mainly around the central vein was also observed in the AME treated group (Figure 2). Mice in this group showed aggressive behavior manifested by biting each other.

Group 1	Normal control	The animals received only distilled water for 7 days
Group 2	AM control	The animals were treated with 100 mg/kg of aqueous AM extract for 7 days
Group 3	BF control	The animals received distilled water for 7 days followed by an intraperitoneal injection of 1.5×10^9 CFU of bacterium *B. firmus*
Group 4	Asp control	The animals received distilled water for 7 days. On day 7, animals were given 100 mg/kg of aspirin two hours prior to intraperitoneal injection of 1.5×10^9 CFU of *B. firmus*
Group 5	AM1 treated	The animals were treated with 100 mg/kg of AM aqueous extract for 7 days. On day 7, animals were injected with 1.5×10^9 CFU of *B. firmus*
Group 6	AM2 treated	The animals received distilled water for 7 days. On day 7, animals were given 100 mg/kg AM water extract 2 hours prior intraperitoneal injection of 1.5×10^9 CFU of *B. firmus*
Group 7	AM-Asp treated	The animals received distilled water for 7 days. On day 7, animals were given 100 mg/kg AM extract and 100 mg/kg aspirin 2 hours prior intraperitoneal injection of 1.5×10^9 CFU *B. firmus*

Table 1: The animals were sacrificed 3 days after treatment.

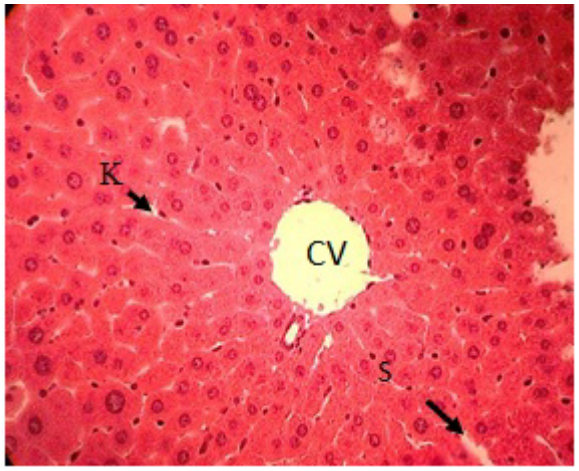

Figure 1: A section of normal mouse liver showing normal architecture of the liver: central vein (CV), hepatocytes arranged in the form of cords. Cords are separated by sinusoids (S) with Kupffer cells (K) (H & E x400).

Treating mice with either AME alone or aspirin alone two hours prior to bacterial infection led to lower inflammatory response as illustrated by a decrease in hepatic parenchymal distress, hydropic degeneration, and absence of necrosis (Figures 5 and 6).

Furthermore, AME and aspirin showed a synergistic effect. Liver of mice that were co-treated with AME and aspirin two hours prior to bacterial injection almost appeared normal. Hepatocytes were arranged in the form of cords, rounded in a polyhedral shape and radiate in peripheral cords (Figure 7).

Effect of AME on glycogen levels in liver

Histopathology of the hepatocytes of control animals and those co-treated with AME and aspirin two hours prior to bacterial infection showed typical normal distribution of glycogen (Figures 8 and 9).

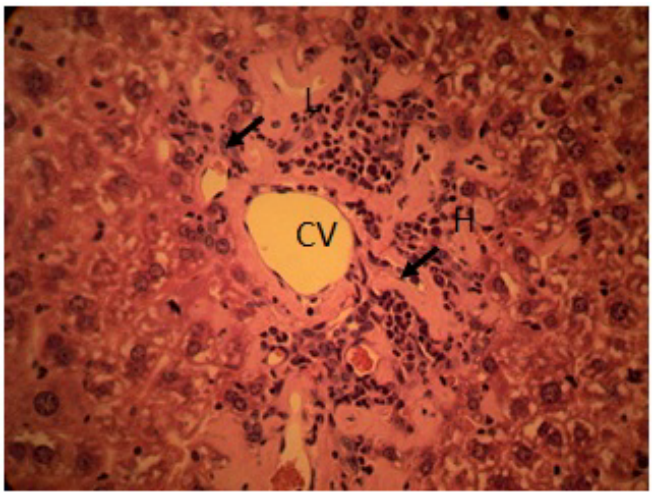

Figure 2: A section of liver from mice that were pre-treated with AME 100 mg/kg for seven day showing lymphocytes infiltration (L), hydropic degeneration of hepatocytes and hyalinosis (H) around central vein (CV) (H & E x400).

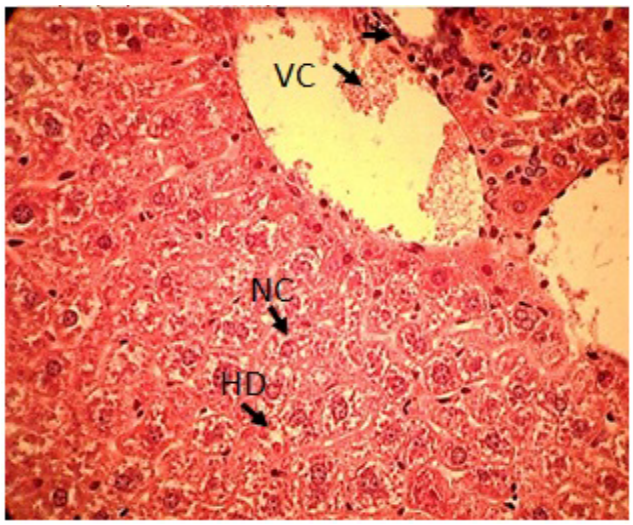

Figure 3: A section of liver from mice injected intra-peritoneally with 1.5 × 10^9 *B. firmus* showing hydropic degeneration of hepatocytes (HD), vascular congestion (VC), hepatic necrosis (NC) and lymphocytes infiltration (H & E x400).

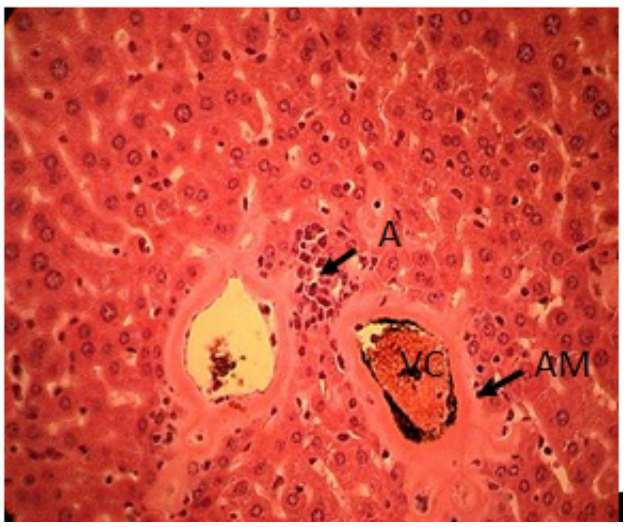

Figure 4: A section of liver from mice intra-peritoneally injected with 1.5 × 10^9 *B. firmus*. Mice were pre-treated with AME 100 mg/kg for seven day prior to bacterial injection showing hepatocytes atrophy (A), vascular congestion (VC) and amyloidosis (AM) around the central vein (H & E x400).

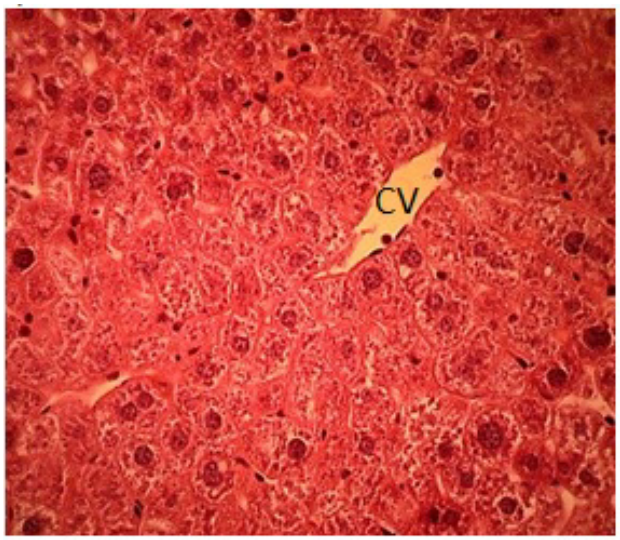

Figure 5: A section of liver from mice intra-peritoneally injected with 1.5 × 10^9 *B. firmus*. Mice were pre-treated with AME 100 mg/kg two hours prior to bacterial injection showing decrease in congestion and absence in necrosis (H & E x400).

On the other hand, mice that were treated with AME alone (Figure 10) showed complete depletion of liver glycogen. The rest of the groups showed re-distribution the glycogen mainly around the central vein (Figures 11-14).

Effect of AME and *B. firmus* on liver amyloidosis

Furthermore, infected mice that were pre-treated with AME for 2 hours (Figure 15) or 7 days prior to bacterial injection (Figure 16) showed mild and moderate deposits of amyloids respectively around the portal vein as compared to control (Figure 17). Surprisingly, neither AME alone nor bacterial infection alone caused any deposits and they were comparable to normal (Figures 18 and 19).

Effect of AME on kidney's histology

Similarly, histopathology of the kidney of animals in the control group and those co-treated with AME and aspirin two hours prior to bacterial infection showed typical renal architecture, normal glomeruli (NG), normal renal tubules (Figures 20 and 21).

While all other groups, i.e., group 2, 3, 4, 5, and 6, revealed histopathological changes represented by dilation of degeneration of convoluted tubules; atrophy of some glomeruli and hypercellularity of others; and in some cases, hemorrhages or congestion (Figures 22-26). The degenerative tubules showed cytolysis, loss of the proximal tubular brush border and tubular irregularity. Lymphocytes infiltration was also observed.

Discussion and Conclusion

Annona muricata is an evidently beneficial plant known for its diverse biological activities as antimicrobial, antidiabetic,

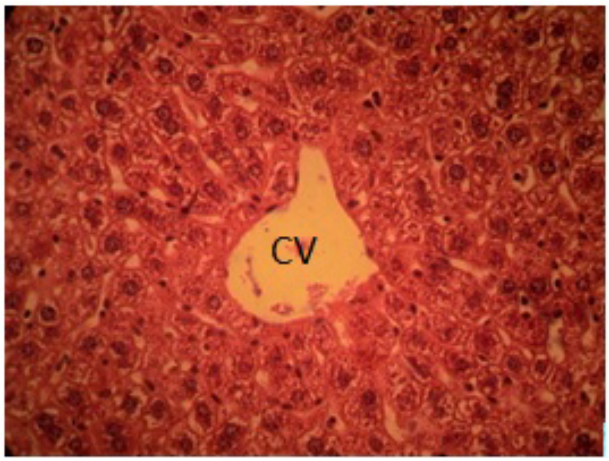

Figure 6: A sectional representative of liver from mice intra-peritoneally injected with 1.5 × 10^9 *B. firmus*. Mice were pretreated with 100 mg/kg aspirin two hours prior to bacterial injection showing decrease in hydropic degeneration, decrease in inflammation and absence in necrosis (H & E x400).

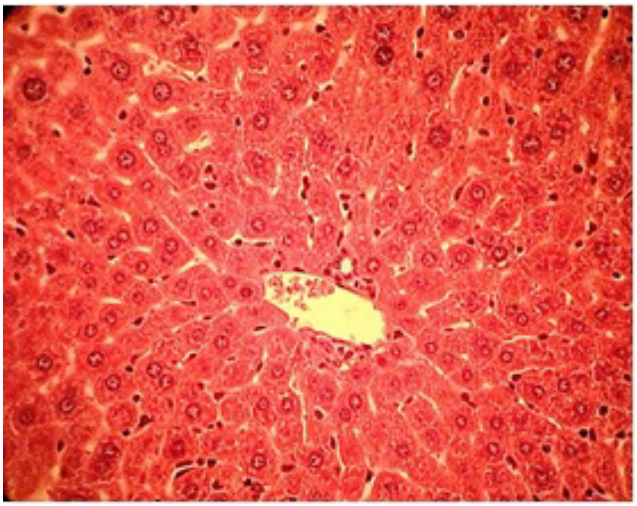

Figure 7: A section of liver from mice intra-peritoneally injected with 1.5 × 10^9 *B. firmus*. Mice were pre-treated with AME 100 mg/kg and 100 mg/kg aspirin two hours prior to bacterial injection showing significant improvement of inflammatory degeneration and necrosis (H & E x400).

anticancerous, noniceptive and others as reviewed by Arthur et al. The anti-inflammatory effect of both the aqueous and ethanolic leaves extracts was shown by many references [27-30].

We show in our study that aqueous AME caused severe hepatotoxicity when used over a period of seven consecutive days even with a dose proven to be safe (Figure 2). Furthermore, infection with 5 × 10^9 cfu *B. firmus* following AME administration increased hepatotoxicity and nephropathicity (Figures 3, 11 and 22). They both caused deranged architecture of hepatocyte with global moderate hydropic degeneration (vacuolar degeneration was seen by some cells) with accumulation of lymphocytes around central vein and bile duct indicating inflammation. Furthermore, pre-treatment with AME extract for 7 days only showed hyalinosis accumulating mainly around the hepatic vein that was not seen in other groups (Figure 3) along with glycogen depletion. Our results confirm those obtained by Ezejindu et al. [31].

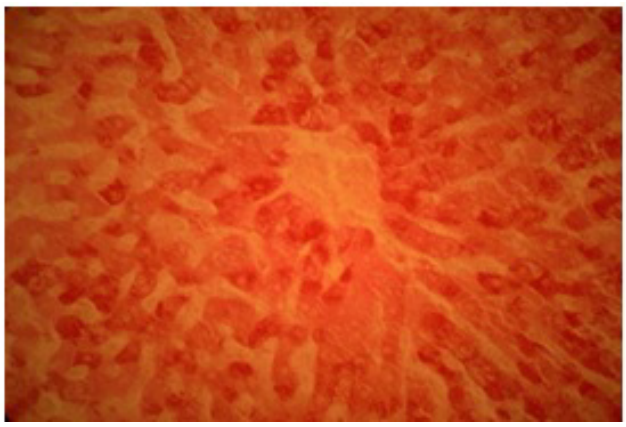

Figure 8: A section of normal mouse liver showing typical normal distribution of glycogen in the hepatocytes (Best carmine x400).

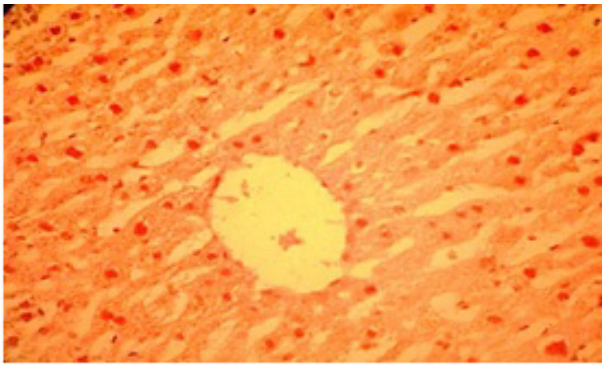

Figure 9: A section of livers from mice co-treated with 100 mg/kg AME + 100 mg/kg aspirin two hours prior to infection with 1.5 × 10^9 *B. firmus* showing typical normal distribution of glycogen in the hepatocytes (Best carmine x400).

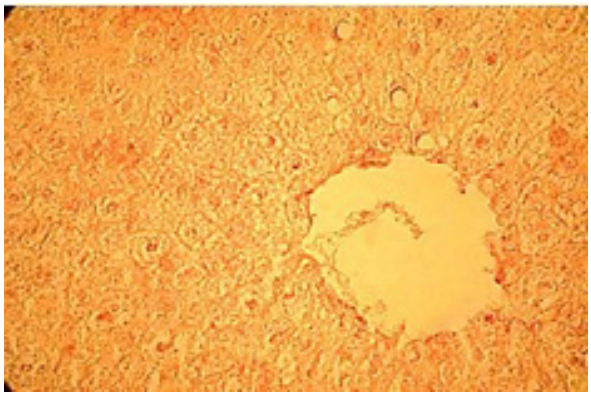

Figure 10: A section of liver from mice pre-treated with AME 100 mg/kg for seven day showing depletion of liver glycogen storage in the hepatocytes (Best carmine x400).

However, two hours pre-treatement of AME alone or aspirin alone prior to bacterial infection was slightly protective. It led to partial restoration of the normal architecture of the hepatocytes where minimized hepatic parenchymal distress and derangement of sinusoids was seen with disturbance of glycogen distribution. This minimized effect could be due to the antibacterial effect exhibited by available

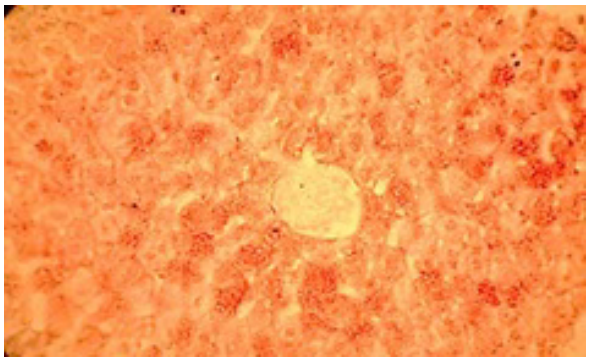

Figure 11: A section of liver from mice intra-peritoneally injected with 1.5 × 10^9 *B. firmus* showing re-distribution of the glycogen in the hepatocytes around central vein (Best carmine x400).

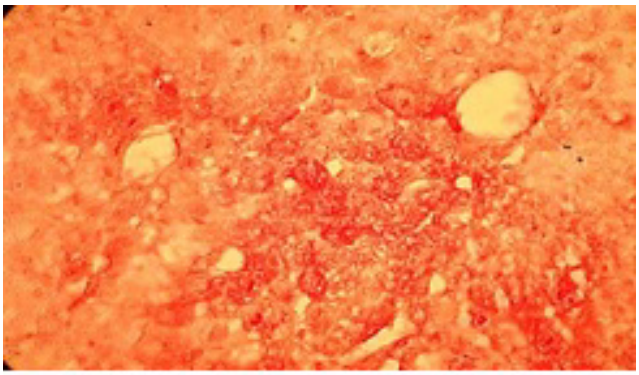

Figure 12: A section of liver from mice intra-peritoneally injected with 1.5 × 10^9 *B. firmus*. Mice were pretreated with 100 mg/kg aspirin two hours prior to bacterial injection showing distribution of the glycogen in the hepatocytes around central vein (Best carmine x400).

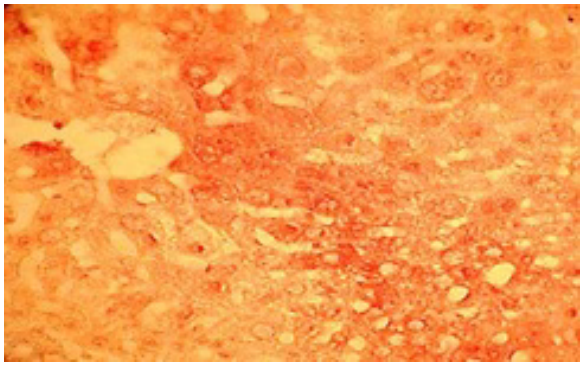

Figure 13: A section of liver from mice intra-peritoneally injected with 1.5 × 10^9 *B. firmus*. Mice were pre-treated with AME 100 mg/kg for seven day prior to bacterial injection showing re-distribution of the glycogen in the hepatocytes around central vein (Best carmine x400).

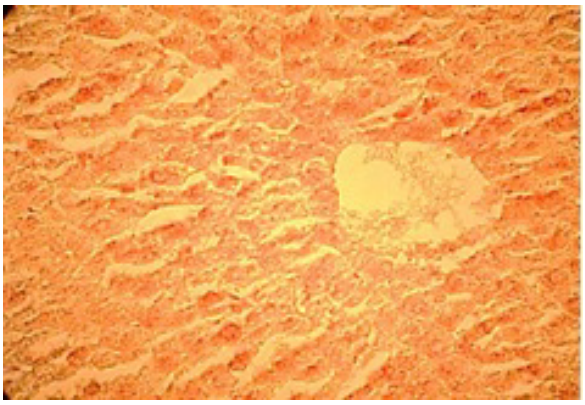

Figure 14: A section of liver from mice intra-peritoneally injected with 1.5 × 10^9 *B. firmus*. Mice were pre-treated with AME 100 mg/kg 2 hours prior to bacterial injection showing re-distribution of the glycogen in the hepatocytes around central vein (Best carmine x400).

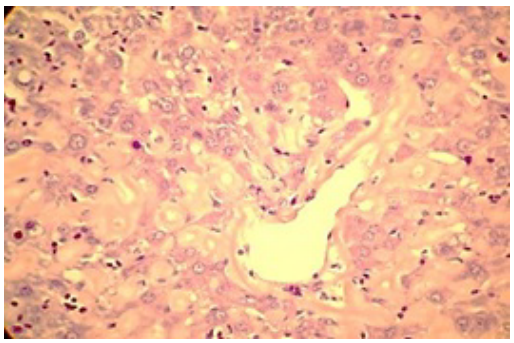

Figure 15: A section of liver from mice that were pre-treated with 100 mg/kg AME 2 hours prior to bacterial injection showing mild deposits of amyloidosis around the portal vein (Congo red, x400).

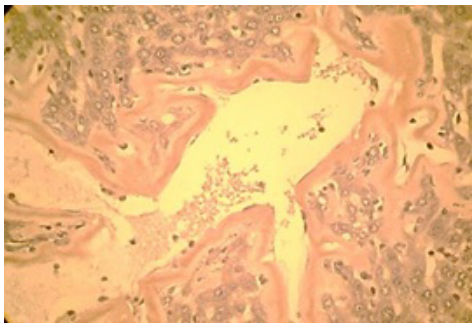

Figure 16: A section of liver from mice that were pre-treated with 100 mg/kg AME 7 days prior to bacterial injection showing moderate deposits of amyloidosis around the portal vein (Congo red, x400).

tannins [32] in AM leaves as shown by others [32-34] and the anti-inflammatory effect of isodesacetyluvaricin, an *Annona muricata* acetogenin that mimics aspirin inhibition of cyclo-oxygenase 2 (COX2) [35-37].

Furthermore, the co-administration of both AME with aspirin prior to bacterial infection seems to have restorative effect on the hepatocytes where normal architecture as observed (Figures 7 and 9). This indicates that AME and aspirin exerted a synergistic effect that minimized the inflammatory response thus protecting the hepatocytes.

The effect of AME was also seen at the kidney level where histopathological changes represented by dilation of distal and degeneration of convoluted tubules, atrophied and hypercellular glomeruli, hemorrhage and signs of congestion in the renal tissue were observed in mice infected with the bacteria alone, mice treated with AME alone

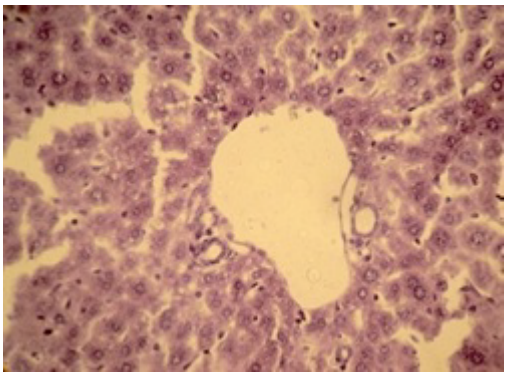

Figure 17: A section of normal mouse liver (Congo red, x400).

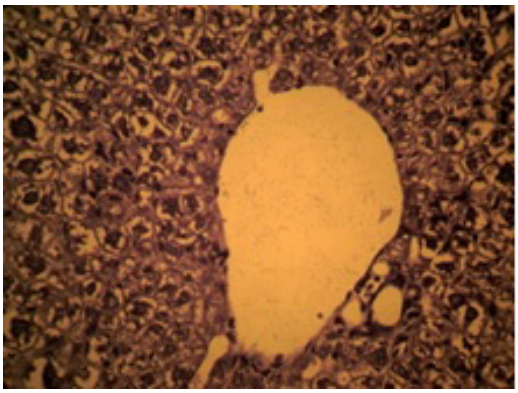

Figure 18: A section of liver from mice intra-peritoneally injected with 1.5×10^9 *B. firmus* (Congo red, x400).

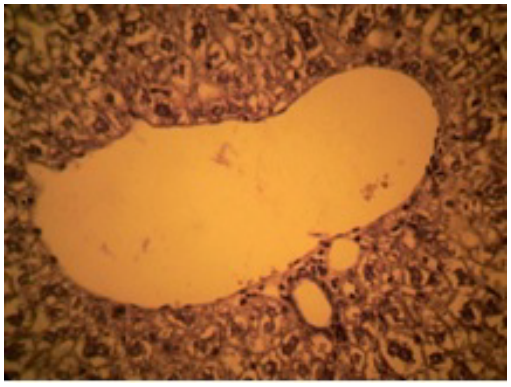

Figure 19: A section of liver from mice were pre-treated with AME 100 mg/kg for seven days (Congo red, x400).

and mice infected with bacteria after 7 days or 2 hours pretreated with AME (Figures 22, 25 and 26) further confirming the results obtained by Ezejindu et al and other [38,39]. However, mice that were co-treated with 100 mg/kg AME and 100 mg/kg aspirin two hours prior to bacterial injection had normal architecture normal kidneys identical to our normal control proving further a synergistic role of AME and aspirin (Figures 23 and 24).

AME also had an effect on the liver glycogen content. Liver glycogen was completely depleted in mice that were treated for 7 days with AME extract implying that AME components may be causing an anorexic

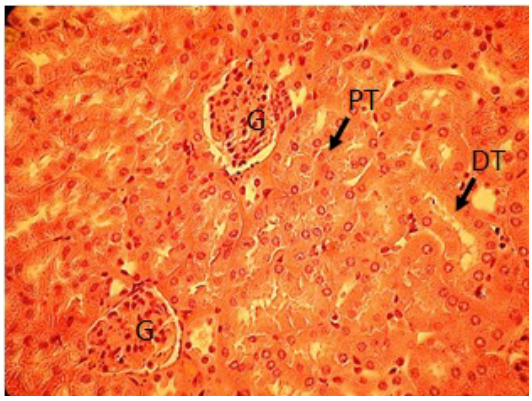

Figure 20: A section of normal mouse kidney showing normal architecture of the kidney. Proximal tubule (PT), distal tubules (DT), glomerulus (G) (H & E x400).

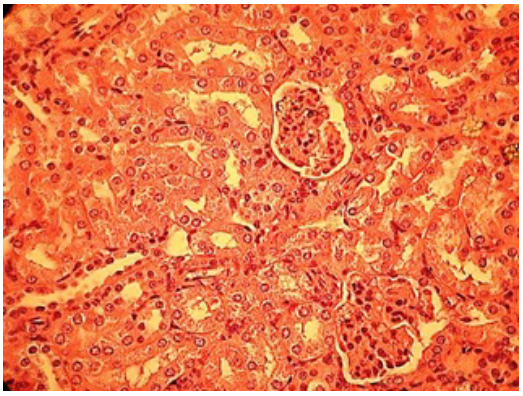

Figure 21: A section of kidney from mice that were pre-treated with 100 mg/kg AME and 100 mg/kg aspirin 2 hour prior to injection with 1.5×10^9 *B. firmus* showing normal architecture of the kidney (H & E x400).

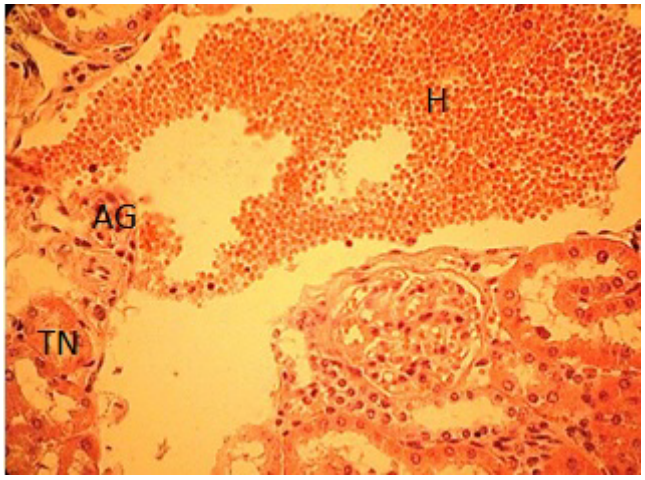

Figure 22: A section of kidney from mice intra-peritoneally injected with 1.5×10^9 *B. firmus* showing atrophied glomeruli (AG) and hemorrhagic foci (H) with tubular necrosis (TN) (H & E x400).

effect and/or an alteration in energy metabolism. Glycogen depletion may suggest an increase in energy utilization and this is in agreement with studies showing that AME has an anti-diabetic effect [22].

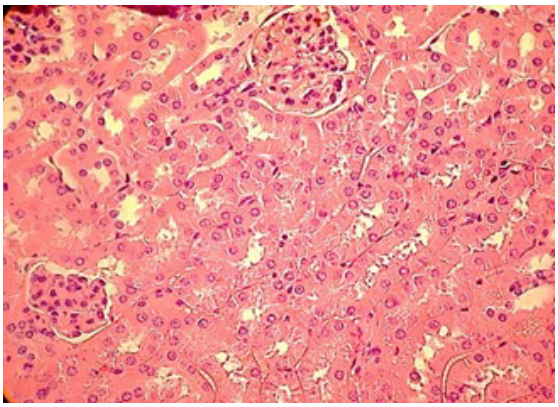

Figure 23: A section of kidney from mice pretreated with 100 mg/kg aspirin 2 hours prior to injection with 1.5 × 10^9 *B. firmus* showing a slight degeneration of the proximal convoluted tubules and distal tubules (H & E x400).

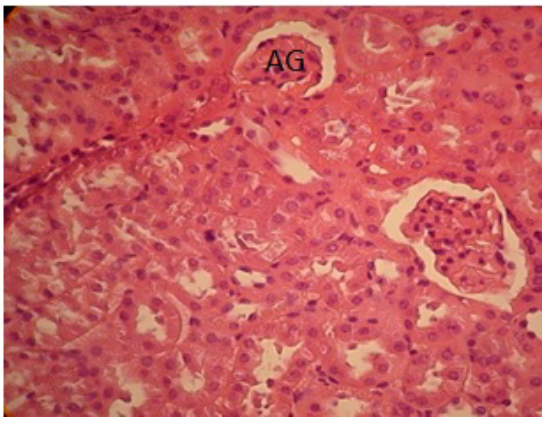

Figure 24: A section of kidney from mice that were pre-treated with 100 mg/kg AME 2 hours prior to injection with 1.5 × 10^9 *B. firmus* showing improvement in inflammation and tubular degeneration (H & E x400).

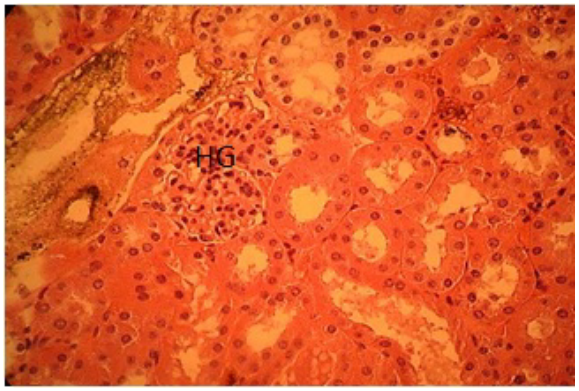

Figure 25: A section of kidney from mice that were pre-treated with AME 100 mg/kg for seven days showing hypercellular glomeruli (HG), hemorrhage and a slight degeneration of the proximal convoluted tubules and distal tubules (H & E x400).

Hence, our study shows that AME extract alone, *B. furmis* alone, or their combinations exert a toxic effect on both liver and the kidneys of albino mice and that their effects can be rectified in the presence of aspirin where it caused a reduction in the inflammatory level and attenuation of leukocytes infiltration [40].

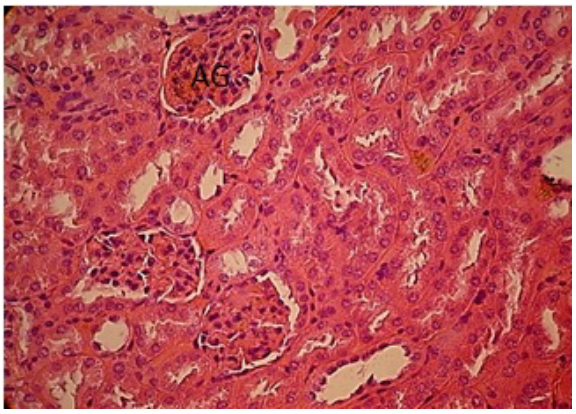

Figure 26: A section of kidney from mice that were pre-treated with 100 mg/kg AME for 7 days prior to injection with 1.5 × 10^9 *B. firmus* showing hypercellularity of glomeruli, and slight degeneration of proximal convoluted tubules (H & E x400).

Toxicity of AME was also proven in our preliminary study as administration of 600 mg/kg for two consecutive days killed the mice confirming the toxic inflammatory effect seen after seven days of 100 mg/kg AME treatment. The hepato-toxicity of *Annona muricata* leaf extract may be due to the presence of acetogenins in the leaves that cause liver and kidney toxicity via increasing calcium concentration, ROS production, and Bax expression and Bax/Bcl-2 ratio [41].

The effect was reversed with aspirin administration. Combination of aspirin and AME abolished as well the effect of AME alone. Hence, further investigation to determine if aspirin masks the effect of AME in a dose dependent manner should be performed.

Finally, liver and kidney sepsis due to different infecting and chemical agents is a serious problem all over the world. Although *B. firmus* is consider as a non-toxic gram-positive bacterium, and was shown to have immunomodulatory effects in mice [42,43] , pigs [44], and nematicide [45]. Our results show that *B. firmus* can be hepatotoxic (Figure 3) and nephropathic (Figure 22) in mice when disseminated intraperitonealy. In addition, it can lead to formation of amyloids deposits around the portal vein in presence of AME (Figure 17). As a result, our study showed that *B. firmus* is a potentially pathogenic microorganism and its pathogenicity needs further investigation.

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